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(54) Title: **METHODS FOR PREVENTING INHIBITION OF NUCLEIC ACID SYNTHESIS BY PYROPHOSPHATE**

## (57) Abstract

Methods for preventing inhibition of nucleic acid synthesis by pyrophosphate are disclosed. More specifically, the present invention concerns inhibiting or preventing pyrophosphorolysis in sequencing and amplification of nucleic acid molecules. According to the present invention, an enzyme which is a pentosyltransferase, a phosphotransferase with an alcohol group as acceptor, a nucleotidyltransferase, or a carboxy-lyase is added to the reaction which serves to remove pyrophosphate from the reaction mixture.

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## Methods for Preventing Inhibition of Nucleic Acid Synthesis by Pyrophosphate

### *Background of the Invention*

#### 5      *Field of the Invention*

10      The present invention relates generally to methods for nucleic acid synthesis. Specifically, the present invention relates to DNA synthesis via a primer extension reaction and methods for RNA synthesis. In particular, the invention relates to methods for avoiding the inhibiting effects of pyrophosphate on RNA synthesis and primer extension DNA reactions, for example, polymerase chain reactions (PCRs) and sequencing reactions.

#### *Background of the Invention*

15      It has been recognized that pyrophosphorolysis, where an oligonucleotide is reduced in length, is detrimental to primer extension reactions. The pyrophosphorolysis is caused by the availability of pyrophosphate. For example, PCR is inhibited by the addition of pyrophosphate even at very low concentrations. According to U.S. Patent 5,498,523, this pyrophosphorolysis can be prevented by providing an agent, for example, a pyrophosphatase, capable of removing pyrophosphate. Addition of pyrophosphatase to a PCR greatly  
20      enhances the progress of the reaction and provides superior results compared to the reaction without a pyrophosphatase. See U.S. Patent No. 4,800,159. Similarly, the addition of a pyrophosphatase to a sequencing reaction provides more uniformity in intensities of bands formed in a polyacrylamide gel used to identify products of the sequencing reaction. This uniformity is due to prevention  
25      of degradation of specific DNA products by pyrophosphorolysis. See also, Tabor, S. and Richardson, C.C., *J. Biol. Chem.* 265:8322 (1990); U.S. Patent No. 4,962,020; and Ruan, C.C. *et al.*, *Comments* 17(1):1 (1990).

Each product or band in a dideoxy sequencing experiment is a polynucleotide complementary to the template and terminated at the 3' end in a

base-specific manner with a dideoxynucleotide. The dideoxy stabilizes the product, preventing further polymerization of the polynucleotide. However, in certain regions of the template, the bands, especially after prolonged reaction, will reduce in intensity or completely disappear ("drop-out" bands). A drop-out may not be readily detected by the operator, leading to errors in the interpretation of the data either by a human or computer-driven analyzer. Since this phenomenon is stimulated by inorganic pyrophosphate, the effect is presumably due to pyrophosphorolysis (reverse polymerization), not 3'-exonucleolytic activity. It is hypothesized that DNA polymerase idling at the end of these terminated products and in the presence of sufficient pyrophosphate will remove the dideoxynucleotide, then extend from the now free 3'-hydroxyl end to another dideoxy termination. In effect, the bands are converted to longer polynucleotides/bands. Removal of pyrophosphate as it is generated in the polymerization reaction eliminates this problem.

Researchers have used a series of enzyme reactions coupled to pyrophosphate generation to measure DNA polymerase activity. In the first (P. Nyren, *Anal. Biochem.* 167:235 (1987)), Nyren used ATP: sulfate adenylyltransferase to convert pyrophosphate and adenosine 5'-phosphosulfate to ATP and sulfate ion. The ATP was used to make light with luciferase. In the second (J.C. Johnson *et al.*, *Anal. Biochem.* 26:137 (1968)), the researchers reacted the pyrophosphate with UDP-glucose in the presence of UTP: glucose-1-phosphate uridylyltransferase to produce UTP and glucose-1-phosphate. In two more steps, polymerase activity was measured spectrophotometrically by the conversion of NADP to NADPH. While these articles describe the use of ATP: sulfate adenylyltransferase and UTP: glucose-1-phosphate uridylyltransferase in measuring DNA polymerase activity, they do not describe their use to prevent or inhibit pyrophosphorolysis in nucleic acid synthesis reactions.

### *Summary of the Invention*

A number of naturally-occurring enzymes use pyrophosphate as a substrate, including certain transferases, kinases and lyases. By coupling the reaction catalyzed by one of these enzymes to the polymerase reaction, pyrophosphate will not build up, preventing pyrophosphorolysis in nucleic acid synthesis reactions. Thus, the present invention relates to a method of inhibiting or preventing pyrophosphorolysis during synthesis of a nucleic acid molecule, said method comprising

- (a) combining one or more nucleotides and a nucleic acid template;
- (b) incubating the one or more nucleotides and nucleic acid template together with a polymerase and an enzyme selected from the group consisting of a pentosyltransferase, a phosphotransferase with alcohol group as acceptor, a nucleotidyltransferase, and a carboxy-lyase, under conditions sufficient to form a second nucleic acid molecule complementary to all or a portion of the nucleic acid template.

The method of the invention more specifically relates to a method of inhibiting or preventing pyrophosphorolysis, said method comprising

- (a) combining a primer with a nucleic acid template under conditions sufficient to form a hybridized product; and
- (b) incubating said hybridized product in the presence of (i) one or more nucleotides, (ii) a polymerase, and (iii) an enzyme selected from the group consisting of a pentosyltransferase, a phosphotransferase with alcohol group as acceptor, a nucleotidyltransferase, and a carboxy-lyase under conditions sufficient to synthesize a second nucleic acid molecule complementary to all or a portion of said nucleic acid template.

Specifically, the method of the present invention relates to inhibition of pyrophosphorolysis in the synthesis of DNA and RNA molecules using the appropriate nucleotides and polymerases (dNTP's/rNTP's and DNA polymerase/ RNA polymerase).

In particular, the present invention may be used in primer extension reactions to prevent the inhibition of nucleic acid synthesis during amplification and may be used to prevent band drop out in sequencing reactions. Thus, the invention relates to a method to prevent inhibition of nucleic acid synthesis during amplification of a double stranded nucleic acid molecule comprising

5 (a) providing a first and second primer, wherein said first primer is complementary to a sequence at or near the 3' termini of the first strand of said nucleic acid molecule and said second primer is complementary to a sequence at or near the 3' termini of the second strand of said nucleic acid molecule;

10 (b) hybridizing said first primer to said first strand and said second primer to said second strand in the presence of (i) a polymerase, and (ii) an enzyme selected from the group consisting of a pentosyltransferase, a phosphotransferase with an alcohol group as an acceptor, a nucleotidyltransferase and a carboxy-lyase under conditions such that a third nucleic acid molecule complementary to said first strand and a fourth nucleic acid molecule complementary to said second strand are synthesized;

15 (c) denaturing said first and third strand and said second and fourth strand; and

(d) repeating steps (a) to (c) one or more times.

20 The present invention also relates to a method of sequencing a DNA molecule comprising:

(a) combining a primer with a first DNA molecule under conditions sufficient to form a hybridized product;

25 (b) contacting said hybridized product with nucleotides, a DNA polymerase, an enzyme selected from the group consisting of a pentosyltransferase, a phosphotransferase with an alcohol group as acceptor, a nucleotidyltransferase and a carboxy-lyase; and a terminator nucleotide to give a reaction mixture;

30 (c) incubating the reaction mixture under conditions sufficient to synthesize a random population of DNA molecules complementary to said first

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DNA molecule, wherein said synthesized DNA molecules are shorter in length than said first DNA molecule and wherein said synthesized DNA molecules comprise a terminator nucleotide at their 3' termini; and

(d) separating said synthesized DNA molecules by size so that at least a part of the nucleotide sequence of said first DNA molecule can be determined.

The invention also relates to a solution for use in nucleic acid synthesis, amplification or sequencing, comprising

(a) an enzyme selected from the group consisting of a pentosyltransferase, a phosphotransferase with alcohol group as acceptor, a nucleotidyltransferase, and a carboxy-lyase;

(b) a substrate which is capable of either accepting a phosphate radical from pyrophosphate or effecting transfer of pyrophosphate to give a phosphorylated product when in the presence of said enzyme; and

(c) a polymerase.

Examples of pentosyltransferases according to the present invention include an adenine phosphoribosyltransferase or an orotate phosphoribosyltransferase. Examples of a phosphotransferase with alcohol group as acceptor include a pyrophosphate: glycerol phosphotransferase, a pyrophosphate: serine phosphotransferase, a pyrophosphate: fructose-6-phosphate 1-phosphotransferase or a pyrophosphate: purine nucleoside kinase. Examples of a nucleotidyltransferase include an ATP: sulfate adenylyltransferase, a UTP: glucose-1-phosphate uridylyltransferase or a glucose-1-phosphate adenylyltransferase. Examples of a carboxy-lyase include phosphoenolpyruvate carboxykinase.

The invention also relates to a kit comprising a container means having in close confinement therein two or more container means, wherein a first container means comprises an enzyme selected from the group consisting of a pentosyltransferase, a phosphotransferase with an alcohol group as acceptor, a nucleotidyltransferase, and a carboxy-lyase; and optionally a nucleic acid polymerase. The polymerase may instead be contained in a second container

means. A third container means comprises a substrate which is capable of either accepting a phosphate radical to give a phosphorylated product from pyrophosphate or effecting transfer of pyrophosphate radical when in the presence of the enzyme.

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### ***Brief Description of the Figures***

Fig. 1 depicts a gel showing the effect of UTP: glucose-1-phosphate uridylyltransferase on pyrophosphorolysis in DNA sequencing reactions.

Fig. 2 depicts a gel showing the effect of ATP: sulfate adenylyltransferase on pyrophosphorolysis in DNA sequencing reactions.

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Fig. 3 depicts a gel showing the effect of Pyrophosphate: fructose-6-phosphate 1-phosphotransferase on pyrophosphorolysis in DNA sequencing reactions.

### ***Detailed Description of the Preferred Embodiments***

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The present invention is directed to the recognition that there are a number of enzyme reactions in a cell which utilize the high energy of a phosphodiester link in certain synthetic pathways, such as those described by Kornberg, A. and Baker, T.A., in *DNA Replication*, 2nd ed., W.H. Freeman and Co., New York (1992), p. 68. These involve quite different enzymes and reactions which are distinct from the degradative inorganic pyrophosphorylase, but still prevent pyrophosphorolysis by reducing the level of pyrophosphate in the mixture. Some of the enzyme types are transferases, kinases and lyases.

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The following are official enzyme classes and particular examples of enzymes that may be used in the practice of the invention:

Class EC 2.4.2.-      Pentosyltransferases

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2.4.2.7              Adenine phosphoribosyltransferase

2.4.2.10            Orotate phosphoribosyltransferase



- Class EC 2.7.1.-      Phosphotransferases with an alcohol group as acceptor
- 2.7.1.79      Pyrophosphate: glycerol phosphotransferase
- 2.7.1.80      Pyrophosphate: serine phosphotransferase
- 2.7.1.90      Pyrophosphate: fructose-6-phosphate 1-phosphotransferase
- 5      2.7.1.143      Pyrophosphate: purine nucleoside kinase

- Class EC 2.7.7.-      Nucleotidyltransferases
- 2.7.7.4      ATP: sulfate adenylyltransferase
- 2.7.7.9      UTP: glucose-1-phosphate uridylyltransferase
- 2.7.7.27      ATP: glucose-1-phosphate adenylyltransferase

- 10      Class EC 4.1.1.-      Carboxy-lyases
- 4.1.1.38      Phosphoenolpyruvate carboxykinase

*See, the CRC Handbook of Biochemistry and Molecular Biology: Proteins (Vol. II), Fasman, G.D., ed., 3rd edition, CRC Press, Cleveland, OH (1976), pp. 93-109, for the internationally developed classification system for enzymes.*

- 15      For comparison, inorganic pyrophosphatase belongs to a group very distinct; hydrolases acting on phosphorous-containing acid anhydrides, with an EC classification number of 3.6.1.1.

- 20      There are many closely-related members of the 2.7.7.9 and 2.7.7.27 enzymes types that may be used in the practice of the invention. Likewise, there are many members of the 2.4.2.- enzyme type that are closely related enzymatically that may be used. Such additional enzymes are listed below:

*Additional members of the 2.4.2.- subclass:*

- E.C. 2.4.2.8      Hypoxanthine phosphoribosyltransferase
- E.C. 2.4.2.9      Uracil phosphoribosyltransferase
- 25      E.C. 2.4.2.11      Nicotinate phosphoribosyltransferase
- E.C. 2.4.2.12      Nicotinamide phosphoribosyltransferase
- E.C. 2.4.2.14      Amidophosphoribosyltransferase

- 5 E.C. 2.4.2.17 ATP phosphoribosyltransferase  
E.C. 2.4.2.18 Anthranilate phosphoribosyltransferase  
E.C. 2.4.2.19 Nicotinate-nucleotide pyrophosphorylase (carboxylating)  
E.C. 2.4.2.20 Dioxotetrahydropyrimidine phosphoribosyltransferase  
E.C. 2.4.2.22 Xanthine-guanine phosphoribosyltransferase

*Additional members of 2.7.7.- subclass:*

- 10 E.C. 2.7.7.1 Nicotinamide-nucleotide adenylyltransferase  
E.C. 2.7.7.2 FMN adenylyltransferase  
E.C. 2.7.7.10 UTP:hexose-1-phosphate uridylyltransferase  
E.C. 2.7.7.11 UTP:xylose-1-phosphate uridylyltransferase  
E.C. 2.7.7.13 Mannose-1-phosphate guanylyltransferase  
E.C. 2.7.7.14 Ethanolamine-phosphate cytidylyltransferase  
E.C. 2.7.7.15 Cholinephosphate cytidylyltransferase  
E.C. 2.7.7.18 Nicotinate-nucleotide adenylyltransferase  
15 E.C. 2.7.7.21 tRNA cytidylyltransferase  
E.C. 2.7.7.23 Glucosamine-1-phosphate uridylyltransferase  
E.C. 2.7.7.24 Glucose-1-phosphate thymidylyltransferase  
E.C. 2.7.7.25 tRNA adenylyltransferase  
E.C. 2.7.7.27 Glucose-1-phosphate adenylyltransferase  
20 E.C. 2.7.7.28 Nucleoside-triphosphate-hexose-1-phosphate nucleotidyltransferase  
E.C. 2.7.7.29 Hexose-1-phosphate guanylyltransferase  
E.C. 2.7.7.30 Fucose-1-phosphate guanylyltransferase  
E.C. 2.7.7.32 Galactose-1-phosphate thymidylyltransferase  
E.C. 2.7.7.33 Glucose-1-phosphate cytidylyltransferase  
25 E.C. 2.7.7.34 Glucose-1-phosphate guanylyltransferase  
E.C. 2.7.7.38 3-deoxy-manno-octulosonate cytidylyltransferase  
E.C. 2.7.7.39 Glycerol-3-phosphate cytidylyltransferase

- 5 E.C. 2.7.7.40 D-ribitol-5-phosphate cytidyltransferase  
 E.C. 2.7.7.41 Phosphatidate cytidyltransferase  
 E.C. 2.7.7.42 Glutamate-ammonia-ligase adenylyltransferase  
 E.C. 2.7.7.43 Acylneuraminate cytidyltransferase  
 E.C. 2.7.7.44 Glucuronate-1-phosphate uridylyltransferase  
 E.C. 2.7.7.45 Guanosine-triphosphate guanylyltransferase  
 E.C. 2.7.7.46 Gentamycin 2'-nucleotidyltransferase  
 E.C. 2.7.7.47 Streptomycin 3'-adenylyltransferase  
 E.C. 2.7.7.50 mRNA guanylyltransferase  
 10 E.C. 2.7.7.52 RNA uridylyltransferase  
 E.C. 2.7.7.54 Phenylalanine adenylyltransferase  
 E.C. 2.7.7.55 Anthranilate adenylyltransferase  
 E.C. 2.7.7.57 N-methylphosphoethanolamine cytidyltransferase  
 E.C. 2.7.7.58 (2,3-dihydroxybenzoyl)adenylate synthase  
 15 E.C. 2.7.7.59 [Protein PII] uridylyltransferase

A number of such enzymes have been cloned and expressed in a recombinant host. See, for example, Lador, U.S. *et al.*, *J. Biol. Chem.* 266:16550-16555 (1991) (Pyrophosphate: fructose-6-phosphate 1-phosphotransferase); Leyh, T.S. *et al.*, *J. Biol. Chem.* 263:2409-2416 (1988) (ATP: sulfate adenylyltransferase); Leyh, T.S. *et al.*, *J. Biol. Chem.* 267:10405-10410 (1992) (ATP: sulfate adenylyltransferase); Weissborn, A.C., *et al.*, *J. Bacteriology* 176:2611-2618 (1994) (UTP:glucose-1-phosphate uridylyltransferase); Allen, T. *et al.*, *Mol. Biochem. Parasitol.* 74:99 (1995) (Adenine phosphoribosyltransferase); Vonstein, V. *et al.*, *J. Bacteriol.* 177:4540 (1995) (Orotate phosphoribosyltransferase); Charng, Y.Y. *et al.*, *Plant Mol. Biol.* 20:37 (1992) (Glucose-1-phosphate adenylyltransferase); Kim, D.J. and Smith, S.M., *Plant Mol. Biol.* 26:423 (1994) (Phosphoenolpyruvate carboxykinase); Jiang, Y. *et al.*, *Exp. Parasitol.* 82:73 (1996) (Hypoxanthine-guanine

phosphoribosyltransferase); Pla, J. *et al.*, *Gene* 165:115 (1995) (ATP phosphoribosyltransferase); Feldman, R.C. *et al.*, *Infect. Immun.* 60:166 (1992) (Uracil phosphoribosyltransferase); Vinitsky, A., *J. Bacteriol.* 173:536 (1991) (Nicotinate phosphoribosyltransferase); Ludin, K.M. *et al.*, *Curr. Genet.* 25:465 (1994) (Amidophosphoribosyltransferase); Rose, A.B. *et al.*, *Plant Physiol.* 100:582 (1992) (Anthranilate phosphoribosyltransferase); Hughes, K.T. *et al.*, *J. Bacteriol.* 175:479 (1993) (Quinolate phosphoribosyltransferase); Jagadeeswaran, P. *et al.*, *Gene* 31:309 (1984) (Xanthine-guanine phosphoribosyltransferase); Nakagawa, S., *Biosci. Biotech. Biochem.* 59:694 (1995) (FMN adenylyltransferase); Marolda, C.L. and Valvano, M.A., *J. Bacteriol.* 175:148 (1993) (Mannose-1-phosphate guanylyltransferase); Kalmar, G.B., *Proc. Natl. Acad. Sci. USA* 87:6029 (1990) (Choline phosphate cytidylyltransferase); Muller-Rober, B. *et al.*, *Plant Mol. Biol.* 27:191 (1995) (Glucose-1-phosphate adenylyltransferase); Shanmugam, K. *et al.*, *Plant Mol. Biol.* 30:281 (1996) (tRNA nucleotidyltransferase); Zapata, G.A. *et al.*, *J. Biol. Chem.* 264:14769 (1989) (Acylneuraminate cytidylyltransferase); and Vakylenko, S.B. *et al.*, *Antibiot. Khimioter.* 38:25 (1993) (Gentamycin 2'-nucleotidyltransferase).

Preferred enzymes which may be used in the practice of the invention are thermostable, that is, they have been isolated from thermophilic organisms or from recombinant host cells that have been transformed with DNA coding for the thermostable enzyme and derived from the thermophilic organisms. Typically, the thermostable enzymes can withstand temperatures above about 70° C to about 100°C for at least about a minute without losing substantially its enzymatic activity. Most preferably, the enzymes are obtained from extreme thermophiles and the thermostable enzyme is used in high temperature cycling reactions (e.g. PCR). Examples of such thermostable enzymes include phosphofructokinase from *Thermoproteus tenax* (Siebers, B. and Hensel, R., *FEMS Microbiol. Lett.* 111:1-8 (1993)); phosphofructokinase from *Bacillus stearothermophilus* (Zhu, X. *et al.*, *Biochem.* 34:2560-5 (1995))(the organism is not an extreme thermophile, the

optimal performance with this enzyme will be in the range 60-65°C)); uridylyltransferase from *Methanococcus jannaschii* (Bult, C.J. *et al.*, *Science* 273:1058-1072 (1996) (optimum temperature near 85°C)); orotate phosphoribosyltransferase from *Thermus thermophilus* (Yamagishi, A *et al.*, *Appl. Environ. Microbiol.* 62:2191-2194 (1996)); and uracil phosphoribosyltransferase from *Bacillus caldolyticus* (Ghim, S.Y. and Neuhard, J. *et al.*, *J. Bacteriol.* 176:3698-707 (1994)).

Of course, it is necessary to also employ a substrate which is capable of either accepting a phosphate radical to give a phosphorylated product from pyrophosphate or effecting transfer of pyrophosphate radical when in the presence of the enzyme.

Examples of such enzyme/substrate combinations are shown in the following Table.

Enzyme	Substrate
Adenine phosphoribosyltransferase	AMP
Orotate phosphoribosyltransferase	Orotidine 5'-phosphate
Pyrophosphate: glycerol phosphotransferase	Glycerol
Pyrophosphate: fructose-6-phosphate 1-phosphotransferase	D-Fructose-6-phosphate*
Pyrophosphate: purine nucleoside kinase	A purine nucleoside
ATP: sulfate adenylyltransferase	Adenosine 5'-phosphosulfate
UTP: glucose-1-phosphate uridylyltransferase	Uridine 5'-diphosphoglucose
Glucose-1-phosphate adenylyltransferase	ADP-glucose
Phosphoenolpyruvate carboxykinase	Oxaloacetate

\* Fructose-2,6-diphosphate may also be added, not as a substrate, but as a stimulator of enzyme activity.

Use of the enzyme/substrate combinations according to the present invention provide a method for preventing pyrophosphorolysis during nucleic acid synthesis, amplification or sequencing. Thus, in one embodiment, the invention

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relates to a method of inhibiting or preventing pyrophosphorolysis during synthesis of a nucleic acid molecule said method comprising:

(a) combining a primer with a first nucleic acid (DNA or RNA) template to give a hybridized product; and

5 (b) incubating the hybridized product in the presence of (i) one or more nucleotides, (ii) a polymerase (DNA polymerase or RNA polymerase) and (iii) an enzyme selected from a group consisting of a pentosyltransferase, a phosphotransferase with an alcohol group as acceptor, a nucleotidyltransferase, and a carboxy-lyase under conditions sufficient to synthesize a second nucleic acid molecule (RNA or DNA) complimentary to all or a portion of said nucleic acid template.

In a second embodiment, the present invention relates to a method of sequencing a DNA molecule comprising:

15 (a) combining a primer with a first DNA molecule under conditions sufficient to give a hybridized product;

(b) contacting the hybridized product with one or more nucleotides, a DNA polymerase, an enzyme selected from the group consisting of a pentosyltransferase, a phosphotransferase with an alcohol group as acceptor, a nucleotidyltransferase, and a carboxy-lyase and a terminator nucleotide, to give a reaction mixture;

20 (c) incubating the reaction mixture under conditions sufficient to synthesize a random population of DNA molecules complimentary to said first DNA molecule, wherein said synthesized DNA molecules are shorter in length than said first DNA molecule and wherein said synthesized DNA molecules comprise a terminator nucleotide at their 3' termini; and

25 (d) separating said synthesized DNA molecules by size so that at least a part of the nucleotide sequence of said first DNA molecule can be determined.

In accordance with the present invention, it is possible to prevent band drop-outs in DNA sequencing. Such band drop-outs occur to varying extents in all known methods using any DNA polymerase. Thus, the invention may be used

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to improve existing sequencing reactions for single-extension using *e.g.*, modified T7 DNA polymerase, or for cycle sequencing, *e.g.*, *Taq*-based cycle sequencing. Further, both radioactive labeling and non-radioactive labeling methods are applicable. For example, *Taq*-based fluorescent sequencing in the Applied Biosystems DNA Sequencer, Model 373 or 377, will suffer errors resulting from pyrophosphorolysis without reducing the pyrophosphate generated in the reaction. Prolonged incubation seems more deleterious for sequencing bands, however, implementing this invention makes the sequencing process more robust.

The dideoxy sequencing method was first described by Sanger *et al.*, *Proc. Natl. Acad. Sci. USA* 74:5463 (1977). Improvements and modifications of the dideoxy sequencing method of Sanger *et al.* which may be used in the practice of the invention are described in US 4,962,020, US 5,498,523, US 4,795,699, 5,173,411, US 5,405,746, US 5,003,059, US 5,409,811, US 5,403,709, US 5,405,747, US 5,411,862, US 5,432,065, US 5,407,799, US 5,525,464, US 5,525,470, US 5,547,859, US 5,503,980, US 5,512,458, US 5,308,751, US 5,106,729, US 5,124,247, US 5,516,633, US 5,484,701, US 4,863,849, US 5,332,666, US 4,851,331, WO96/14434, WO95/20682, WO94/16107, WO95/23236, WO94/03643, WO93/04184, WO93/20232, WO93/05060, CA 1,311,201, and EP 0409 078. *See also* the *M13 Cloning/Dideoxy Sequencing Instruction Manual*, BRL, Gaithersburg, MD 20884 (1980).

In accordance with the present invention, it is also possible to prevent pyrophosphorolysis during amplification of nucleic acid molecules. Thus, the present invention relates to a method of preventing inhibition of nucleic acid synthesis during amplification of double-stranded nucleic acid molecules comprising:

(a) providing a first and second primer, wherein said first primer is complementary to a sequence at or near the 3' termini of the first strand of said nucleic acid molecule and said second primer is complementary to a sequence at or near the 3' termini of the second strand of said nucleic acid molecule;

(b) hybridizing said first primer to said first strand and said second primer to said second strand in the presence of (i) a polymerase, and (ii) an enzyme selected from the group consisting of a pentosyltransferase, a phosphotransferase with an alcohol group as an acceptor, a nucleotidyltransferase and a carboxy-lyase, under conditions such that a third nucleic acid molecule complementary to said first strand and a fourth nucleic acid molecule complementary to said second strand are synthesized;

(c) denaturing said first and third strand and said second and fourth strand; and

(d) repeating steps (a) to (c) one or more times.

DNA polymerase enzymes that may be used according to the invention, e.g. dideoxy sequencing and PCR, include the wild type and mutant *Tne* DNA polymerases; Sequenase (T7 DNA polymerase), *Taq* DNA polymerase, Thermo Sequenase, *E. coli* polI and Klenow fragment, AmpliTaq FS™, T5 DNA polymerase and mutants thereof. Patents and patent applications describing these polymerases and others which may be used in the practice of the invention include US 5,270,179 US 5,466,591, US 5,455,170, US 5,374,553, US 5,420,029, US 5,075,216, US 5,489,523, US 5,474,920, US 5,210,036, US 5,436,326, US 5,198,543, US 5,108,892, US 5,192,674, US 5,242,818, US 5,413,926, US 4,767,708, US 5,436,149, US 5,500,363, US 5,352,778, US 5,405,774, US 5,545,552, WO96/14417, EP O 712 927, WO95/27067, WO91/09950, WO96/14405, WO95/14770, WO95/04162, WO92/06202, WO92/06188, EP O 482 714, EP O 701 000, EP O 547, 359, EP O 386 859, EP O 386 858, WO96/10640, and application no. 08/706,702, filed September 6, 1996, entitled "Cloned DNA Polymerases from *Thermotoga maritima* and Mutants Thereof." Preferably, the DNA polymerase is a thermostable DNA polymerase such as *Tne*, *Taq*, or *Tma* and mutants thereof which exhibit little or no discrimination between dideoxynucleoside triphosphates and deoxynucleoside triphosphates, which exhibit little or no 3' to 5' exonuclease activity, and which exhibit little or no 5' to 3' exonuclease activity.



RNA polymerase enzymes that may be used according to the invention include any one of the RNA polymerase I, II or III enzymes that are described, for example, in U.S. Patent Nos. 5,550,035, 5,102,802, 5,122,457, 5,126,251, 4,952,496, 4,766,072, and 5,026,645; WO 95/15380, and WO 94/26911; and EP 647,716. Preferred RNA polymerase enzymes include SP6 RNA polymerase, T3 RNA polymerase and T7 RNA polymerase which are commercially available from Life Technologies, Inc. (Gaithersburg, MD).

Chain terminators for DNA synthesis and sequencing reactions are listed in the following Table.

	Type of nucleoside triphosphate	DNA polymerase tested	Reference
5	ribonucleoside 5'-triphosphate	Calf thymus TdT	Beabealashvilli <i>et al.</i> , <i>Biochim. Biophys. Acta</i> 868:136-144 (1986).
15	ribonucleoside 5'-triphosphate	<i>E. coli</i> DNA Polymerase I	Chidgeavadze <i>et al.</i> , <i>Biochim. Biophys. Acta</i> 868:145-152 (1986).
	arabinonucleoside 5'-triphosphate	Calf thymus TdT Reverse transcriptase	Beabealashvilli <i>et al.</i> , <i>Biochim. Biophys. Acta</i> 868:136-144 (1986).
20	3'-amino-3'-deoxyarabinonucleoside 5'-triphosphate	T4 DNA Polymerase	Chidgeavadze <i>et al.</i> , <i>Biochim. Biophys. Acta</i> 868:145-152 (1986).
	2'-deoxy-2'-aminoribonucleoside 5'-triphosphate	<i>E. coli</i> DNA Polymerase I	Chidgeavadze <i>et al.</i> , <i>Biochim. Biophys. Acta</i> 868:145-152 (1986).
25	3'-azido-2', 3'-dideoxyribonucleoside 5'-triphosphate	Reverse transcriptase	Beabealashvilli <i>et al.</i> , <i>Biochim. Biophys. Acta</i> 868:136-144 (1986).
	3'-azido-2', 3'-dideoxyribonucleoside 5'-triphosphate	<i>E. coli</i> DNA polymerase I	Pyrinova <i>et al.</i> , <i>Molekulyarnaya Biologiya</i> 22:1405-1410 (1988).
30	3'-amino-2', 3'-dideoxyribonucleoside 5'-triphosphate	<i>E. coli</i> DNA Polymerase I Calf thymus DNA Pol. $\alpha$ Rat liver polymerase $\beta$	Chidgeavadze <i>et al.</i> , <i>Nucl. Acids Res.</i> 12:1671-1686 (1984).
35	3'-amino-2', 3'-dideoxyribonucleoside 5'-triphosphate	Calf thymus TdT Reverse transcriptase	Beabealashvilli <i>et al.</i> , <i>Biochim. Biophys. Acta</i> 868:136-144 (1986).

10	Type of nucleoside triphosphate	DNA polymerase tested	Reference
	3'-amino-2', 3'-dideoxyribonucleoside 5'-triphosphate	<i>E. coli</i> DNA Polymerase I Calf thymus DNA Pol. $\alpha$	Chidgeavadze <i>et al.</i> , <i>Biochim. Biophys. Acta</i> 868:145-152 (1986).
5	3'-N-acetyl-amino-2', 3'-dideoxyribonucleoside 5'-triphosphate	Calf thymus TdT Reverse transcriptase	Beabealashvilli <i>et al.</i> , <i>Biochim. Biophys. Acta</i> 868:136-144 (1986).
	3'-N-acetyl-amino-2', 3'-dideoxyribonucleoside 5'-triphosphate	<i>E. coli</i> DNA Polymerase I	Chidgeavadze <i>et al.</i> , <i>Biochim. Biophys. Acta</i> 868:145-152 (1986).
10	3'-fluorescaminyl-2', 3'-dideoxyribonucleoside 5'-triphosphate	Reverse transcriptase	Beabealashvilli <i>et al.</i> , <i>Biochim. Biophys. Acta</i> 868:136-144 (1986).
	3'-fluorescaminyl-2', 3'-dideoxyribonucleoside 5'-triphosphate	<i>E. coli</i> DNA Polymerase I Calf thymus DNA Pol. $\alpha$	Chidgeavadze <i>et al.</i> , <i>Biochim. Biophys. Acta</i> 868:145-152 (1986).
15	3'-N-biotinyl-amino-2', 3'-dideoxyribo-nucleoside 5'-triphosphate	Calf thymus TdT Reverse transcriptase	Beabealashvilli <i>et al.</i> , <i>Biochim. Biophys. Acta</i> 868:136-144 (1986).
	3'-N-biotinyl-amino-2', 3'-dideoxyribo-nucleoside 5'-triphosphate	<i>E. coli</i> DNA Polymerase I Calf thymus DNA Pol. $\alpha$	Chidgeavadze <i>et al.</i> , <i>Biochim. Biophys. Acta</i> 868:145-152 (1986).
20	3'-amino-3'-deoxyarabinonucleoside 5'-triphosphate	Calf thymus TdT Reverse transcriptase	Beabealashvilli <i>et al.</i> , <i>Biochim. Biophys. Acta</i> 868:136-144 (1986).
	3'-azido-3'-deoxyarabinonucleoside 5'-triphosphate	Reverse transcriptase	Beabealashvilli <i>et al.</i> , <i>Biochim. Biophys. Acta</i> 868:136-144 (1986).
25	2'-deoxy-3'-O-methylribonucleoside 5'-triphosphate	Reverse transcriptase	Beabealashvilli <i>et al.</i> , <i>Biochim. Biophys. Acta</i> 868:136-144 (1986).
30	2'-deoxy-3'-O-methylribonucleoside 5'-triphosphate	AMV reverse transcriptase	Kutateladze <i>et al.</i> , <i>FEBS Lett.</i> 207:205-212 (1986).
	2',3'-dideoxy-3'-fluororibonucleoside 5'-triphosphate	<i>E. coli</i> DNA Polymerase I AMV reverse transcriptase Calf thymus TdT	Chidgeavadze <i>et al.</i> , <i>FEBS Lett.</i> 183:275-278 (1985).
35	2',3'-dideoxy-3'-fluororibonucleoside 5'-triphosphate	Reverse transcriptase	Beabealashvilli <i>et al.</i> , <i>Biochim. Biophys. Acta</i> 868:136-144 (1986).

	Type of nucleoside triphosphate	DNA polymerase tested	Reference
10	2',3'-dideoxy-3'-fluororibonucleoside 5'-triphosphate	T4 DNA Polymerase	Chidgeavadze <i>et al.</i> , <i>Biochim. Biophys. Acta</i> 868:145-152 (1986).
5	2',3'-dideoxy-2',3'-dideoxyribonucleoside 5'-triphosphate	<i>E. coli</i> polymerase I KF Rat liver DNA polymerase $\beta$ AMV reverse transcriptase RSV reverse transcriptase Calf thymus TdT	Dyatkina <i>et al.</i> , <i>FEBS Lett.</i> 219:151-155 (1987).
	3'-chloro-2',3'-dideoxyribonucleoside 5'-triphosphate	<i>E. coli</i> DNA polymerase I, Rat liver DNA Polymerase $\beta$ AMV reverse transcriptase RSV reverse transcriptase Calf thymus TdT	Krayevsky <i>et al.</i> , <i>Nucleosides and Nucleotides</i> 7:613-617 (1988).
10	3'-methylsulfonamido-2',3'-dideoxy-ribonucleoside 5'-triphosphate	AMV reverse transcriptase	Krayevsky <i>et al.</i> , <i>Nucleosides and Nucleotides</i> 7:613-617 (1988).
15	2',3'-di-O-isopropylideneribonucleoside 5'-triphosphate	AMV reverse transcriptase	Krayevsky <i>et al.</i> , <i>Nucleosides and Nucleotides</i> 7:613-617 (1988).

The term "nucleotide" includes deoxyribonucleoside triphosphates such as dATP, dCTP, dITP, dUTP, dGTP, dTTP, or derivatives thereof. Such derivatives include, for example [ $\alpha$ S]dATP, 7-deaza-dGTP and 7-deaza-dATP. The term "nucleotide" as used herein also refers to ribonucleoside triphosphates (rNTPs) and their derivatives. Illustrated examples of ribonucleoside triphosphates include, but are not limited to, ATP, CTP, GTP, ITP and UTP.

This invention may also be used in methods where improvement of synthesis of nucleic acids by a polymerase is desired and where pyrophosphorolysis is deemed counter-productive. Uses include: polymerase chain reaction, especially 'Long PCR,' and cDNA synthesis. Examples of patents describing these methods include US 4,965,188, US 5,079,352, US 5,091,310, US 5,142,033, US 4,683,195, US 4,683,202, US 4,800,159, US 5,512,462 and US 5,405,776. In the case of cDNA synthesis, a reverse transcriptase polymerase is

incubated with the mRNA template, the deoxynucleoside triphosphates and the enzyme which prevents the build up of pyrophosphate.

5 The invention also relates to a kit comprising a container means such as a box having in close confinement therein two or more container means such as vials, ampules, tubes, jars and the like, each of which contain the materials necessary to carry out the invention. For example, a first container means may comprise an enzyme selected from the group consisting of a pentosyltransferase, a phosphotransferase with an alcohol group as acceptor, a nucleotidyltransferase, and a carboxy-lyase. This first container means may also comprise a DNA or RNA  
10 polymerase. Alternatively, a second container means may comprise the DNA or RNA polymerase. A third container means will comprise a substrate which is capable of either accepting a phosphate radical to give a phosphorylated product from pyrophosphate or effecting transfer of pyrophosphate when in the presence of the enzyme. Other container means may contain other reagents that are  
15 necessary for carrying out dideoxy sequencing or PCR as are well known.

Preferably, the contents of the container means are present at working concentrations (e.g. 1X). Other container means may contain other reagents that are necessary for carrying out dideoxy sequencing or amplification (PCR). Methods for preparing such compositions at working concentrations are described  
20 in application no. 08/689,815, filed August 14, 1996, entitled "Stable Compositions for Nucleic Acid Sequencing and Amplification."

The enzyme and substrate used for reducing pyrophosphate concentration may be mixed directly with the polymerase at their appropriate concentrations, which in turn may be further mixed with reaction buffer and nucleotides. In  
25 general, the enzymes and substrate are present at concentrations sufficient to reduce the level of pyrophosphate in nucleic acid synthesis, amplification or sequencing reactions. Preferably, the enzymes and substrate are present at concentrations which reduce the level of pyrophosphate and, as a result, prevent pyrophosphorolysis (e.g., reduce the inhibition of amplification reactions and/or  
30 reduce or eliminate band drop out in sequencing reactions). Particular

concentrations of the enzyme will vary according to the activity of the enzyme and the temperature of the reaction. Additionally, in high temperature cycling reactions (PCR), it may be necessary to add enzyme after each cycle if the enzyme is inactivated by the cycle temperature. By way of illustration, when the enzyme is UTP: glucose-1-phosphate uridylyltransferase, the polymerase is Sequenase Version 2.0, and the temperature of the reaction is 37°C, the concentration of UTP: glucose-1-phosphate uridylyltransferase may range from about 0.01 U/ $\mu$ l to about 15 U/ $\mu$ l, preferably about 0.15 U/ $\mu$ l. The concentration of the uridine 5'-diphosphoglucose may range from about 10  $\mu$ M to about 0.5 M, preferably about 190  $\mu$ M. If instead the enzyme is ATP: sulfate adenylyltransferase, the concentration of ATP: sulfate adenylyltransferase may range from about 0.001 U/ $\mu$ l to about 2 U/ $\mu$ l, preferably about 0.002 U/ $\mu$ l, and the concentration of adenosine 5'-phosphosulfate may range from about 0.25  $\mu$ M to about 0.5 M, preferably about 5  $\mu$ M. If the enzyme is Pyrophosphate: fructose-6-phosphate 1-phosphotransferase, the concentration of Pyrophosphate: fructose-6-phosphate 1-phosphotransferase may range from about 0.00004 U/ $\mu$ l to about 4 U/ $\mu$ l, and the concentration of fructose-6-phosphate may range from about 10  $\mu$ M to about 0.5 M, preferably about 190  $\mu$ M. The concentration of the stimulatory cofactor fructose-2,6-diphosphate may range from about 0.5 nM to about 100  $\mu$ M, preferably about 50 nM.

Thus, the solution of the present invention is an aqueous and/or buffered liquid containing the components described above. These components are present in the solution at concentrations sufficient to perform their desired function. If the reaction mixture is intended to amplify a target nucleic acid molecule, the reaction mixture will contain the enzyme which reduces the level of pyrophosphate, the substrate which is capable of either accepting a phosphate radical to give a phosphorylated substrate from pyrophosphate or effecting transfer of pyrophosphate when in the presence of the enzyme, a DNA polymerase, all four dNTPs, the one or two oligonucleotide primers having a single stranded region (and optionally a double stranded region) which are capable of annealing to the

target nucleic acid molecule, being extended and thereby amplified. The primer extension reaction may also comprise a chain terminator as described herein, e.g. a dideoxynucleoside triphosphate, which allows for sequencing of the target DNA molecule by the well known Sanger dideoxy sequencing method.

5 In general, the enzymes described in this invention are ubiquitous in nature so different versions of any one enzyme could be obtained from different organisms for different reaction situations. For example, a sub-room temperature reaction may be preferred, where an enzyme from a cryophile may be appropriate. Alternatively, thermostable enzymes may be utilized. Further, many examples of  
10 enzymes using pyrophosphate as a co-substrate are known, so many different versions of this invention are anticipated. For example, the best conditions to enhance Long PCR may not be the same as for DNA sequencing, thus a different enzyme may be needed for each application. The variety of enzymes and sources permits flexibility in optimal design of application of the invention.

15 The invention is based on enzymatic removal of the pyrophosphate concomitant with nucleic acid synthesis. Some enzymes may not be compatible with the reaction environment preferred for nucleic acid synthesis, e.g., pH, monovalent cation concentration, or Tris buffer. Many different enzymes exist which are anticipated to provide the needed reduction of pyrophosphate  
20 concentration. Thus, an appropriate enzyme can be found that would be compatible without requiring compromise of optimal nucleic acid synthesis conditions. Two examples are already provided in the literature (Nyren, *ibid.* and Johnson *et al*, *ibid.*).

25 In the Examples which follow, either UTP: glucose-1-phosphate uridylyltransferase and uridine-5'-diphosphoglucose, or ATP: sulfate adenylyltransferase and adenosine 5'-phosphosulfate, or Pyrophosphate: fructose-6-phosphate 1-phosphotransferase and fructose-6-phosphate, were used in a Sequenase™ reaction incubated for 30 minutes without observing drop-out bands. Without both enzyme and substrate, many drop-out bands are evident in a 30-  
30 minute incubation.

Having now generally described the invention, the same will be more readily understood through reference to the following Examples which are provided by way of illustration, and are not intended to be limiting of the present invention, unless specified.

5

### *Examples*

***Example 1 Use of UTP: glucose-1-phosphate uridylyltransferase to eliminate pyrophosphorolysis in DNA sequencing***

Template and primer sufficient for 7 sequencing reactions were annealed in a 39  $\mu$ l reaction volume by incubating for two minutes at 65°C in a heating block and then slowly cooling the reaction to less than 37°C. The composition of the reaction was: 74 nM M13mp19(+) strand DNA, 90 nM M13/pUC 23 base Forward Sequencing Primer, 72 mM Tris-HCl (pH 7.5), 45 mM NaCl, 18 mM MgCl<sub>2</sub>. Six separate reactions were then radiolabeled by incubating the following 15.5  $\mu$ l reactions at room temperature (23-24°C) for 2 minutes: 26.5 nM M13Mp19(+) strand DNA, 32 nM M13/pUC 23 base Forward Sequencing Primer, 129 mM Tris-HCl (pH 7.5), 32 mM NaCl, 13 mM MgCl<sub>2</sub>, 6.5 mM DTT, 0.32  $\mu$ M (0.32  $\mu$ Ci/ $\mu$ l) [ $\alpha$ -<sup>35</sup>S]dATP, 0.194  $\mu$ M dCTP, 0.194  $\mu$ M 7-deaza-dGTP, 0.194  $\mu$ M dTTP, 0.21 U/ $\mu$ l Sequenase Version 2.0 T7 DNA Polymerase. Additional components were present in the indicated reactions at the concentrations given in the following table:

20

Reaction Number	Concentration of Inorganic Pyrophosphatase (U/ $\mu$ l)	Concentration of uridine-5'-diphosphoglucose* ( $\mu$ M)	Concentration of UTP: glucose-1-phosphate uridylyltransferase (U/ $\mu$ l)**
1	0	0	0
2	0.00004	0	0
3	0	320	0.016
4	0	320	0.0032
5	0	320	0.00065
6	0	0	0.0032

\* uridine-5'-diphosphoglucose (Cat. No. U-4625) Sigma

\*\* UTP: glucose-1-phosphate uridylyltransferase (Cat. No. U-5877) Sigma

Each mixture was then divided into four tubes for completion of base-specific termination reactions. These sets of reactions were incubated for 30 minutes at 37°C and had the following compositions in 6  $\mu$ l reaction volumes: 15.4 nM M13mp19(+) strand DNA, 18.5 nM M13/pUC 23 base Forward Sequencing Primer, 75 mM Tris-HCl (pH 7.5), 40 mM NaCl, 7.5 mM MgCl<sub>2</sub>, 3.8 mM DTT, 0.19  $\mu$ M (0.19  $\mu$ Ci/ $\mu$ l) [ $\alpha$ -<sup>35</sup>S]dATP, 33.3  $\mu$ M dATP, 33.4  $\mu$ M dCTP, 33.4  $\mu$ M 7-deaza-dGTP, 33.4  $\mu$ M dTTP, 0.12 U/ $\mu$ l Sequenase Version 2.0, T7 DNA Polymerase, and 3.3  $\mu$ M ddATP, ddCTP, ddGTP or ddTTP. Additional components were present in the indicated reaction sets at the concentrations given in the following table:



Reaction Number	Concentration of Inorganic Pyrophosphatase (U/ $\mu$ l)	Concentration of uridine-5'-diphosphoglucose ( $\mu$ M)	Concentration of UTP: glucose-1-phosphate uridylyltransferase (U/ $\mu$ l)
1	0	0	0
2	0.00002	0	0
3	0	187	0.0093
4	0	187	0.0019
5	0	187	0.00038
6	0	0	0.0019

Reactions were stopped by adding 4  $\mu$ l of 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF and denatured for 2 minutes at 70°C. Three-microliter aliquots were separated on a 6% TBE-7 M urea sequencing gel. The dried gel was exposed to Kodak BioMAX x-ray film at room temperature for approximately 18 hours.

Results from these six sets of reactions are shown in the photograph of the gel (Fig. 1), where each set are separately displayed, left to right, in the order 1 through 6 as described in the above table. Under conditions where neither UTP: glucose-1-phosphate uridylyltransferase nor inorganic pyrophosphatase were present in the reactions (set 1), certain sequencing bands are either faintly or not visible as indicated by an asterisk on the figure. When UTP: glucose-1-phosphate uridylyltransferase is added at two different enzyme concentrations (sets 3-4), the bands are fully visible. In the presence of a lower concentration of this enzyme (set 5), only partial recovery of the bands is seen. Even though UTP: glucose-1-phosphate uridylyltransferase was present in set 6, the absence of the enzyme substrate uridine 5'-diphosphoglucose prevented the enzyme from reacting with pyrophosphate and providing protection from band loss. The inhibition of pyrophosphorolysis demonstrated in this example is dependent on both enzyme and enzyme substrate and is not the result of a contaminating pyrophosphatase-

type activity. For reference, the action of inorganic pyrophosphatase in protecting from band loss is shown in set 2.

*Example 2 Use of ATP: sulfate adenylyltransferase to eliminate pyrophosphorolysis in DNA sequencing*

5           Template and primer sufficient for 7 sequencing reactions were annealed in a 70  $\mu$ l reaction volume by incubating for two minutes at 65°C in a heating block and then slowly cooling the reaction to less than 37°C. The composition of the reaction was: 41 nM M13mpl9(+) strand DNA, 50 nM M13/pUC 23 base Forward Sequencing Primer, 200 mM Tris-HCl (pH 7.5), 50 mM NaCl, 20 mM  
10   MgCl<sub>2</sub>. Five separate reactions were then radiolabeled by incubating the following 15.5  $\mu$ l reactions at room temperature (23-24°C) for 2 minutes: 26.5 nM M13mpl9(+) strand DNA, 32 nM M13/pUC 23 base Forward Sequencing Primer, 129 mM Tris-HCl (pH 7.5), 32 mM NaCl, 13 mM MgCl<sub>2</sub>, 6.5 mM DTT, 0.32  $\mu$ M (0.32  $\mu$ Ci/ $\mu$ l) [ $\alpha$ -<sup>35</sup>S]dATP, 0.194  $\mu$ M dCTP, 0.194  $\mu$ M 7-deaza-dGTP,  
15   0.194  $\mu$ M dTTP, 0.21 U/ $\mu$ l Sequenase Version 2.0 T7 DNA Polymerase. Additional components were present in the indicated reaction sets at the concentrations given in the following table:

Reaction Number	Concentration of Inorganic Pyrophosphatase (U/ $\mu$ l)	Concentration of adenosine 5'-phosphosulfate* ( $\mu$ M)	Concentration of ATP: sulfate adenylyltransferase ** (U/ $\mu$ l)
1	0	0	0
2	0.00004	0	0
3	0	8.4	0.0016
4	0	8.4	0.0032
5	0	0	0.0032

25   \* adenosine 5'-phosphosulfate (Cat.No.A-5508) Sigma

\*\* ATP: sulfate adenylyltransferase (Cat.No.A-8957) Sigma

Each mixture was then divided into four tubes for completion of base-specific termination reactions. These sets of reactions were incubated for 30 minutes at 37°C and had the following compositions in 6 µl reaction volumes: 15.4 nM M13mpl9(+) strand DNA, 18.5 nM M13/pUC 23 base Forward Sequencing Primer, 75 mM Tris-HCl (pH 7.5), 40 mM NaCl, 7.5 mM MgCl<sub>2</sub>, 3.8 mM DTT, 0.19 µM (0.19 µCi/µl) [ $\alpha$ -<sup>35</sup>S]dATP, 33.3 µM dATP, 33.4 µM dCTP, 33.4 µM 7-deaza-dGTP, 33.4 µM dTTP, 0.12 U/µl Sequenase Version 2.0 T7 DNA Polymerase, and 3.3 µM ddATP, ddCTP, ddGTP or ddTTP. Additional components were present in the indicated reactions at the concentrations given in the following table:

Reaction Number	Concentration of Inorganic Pyrophosphatase (U/µl)	Concentration of adenosine 5'-phosphosulfate (µM)	Concentration of ATP: sulfate adenylyltransferase (U/µl)
1	0	0	0
2	0.00002	0	0
3	0	4.9	0.0093
4	0	4.9	0.0019
5	0	0	0.0019

Reactions were stopped by adding 4 µl of 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF and denatured for 2 minutes at 70°C. Three-microliter aliquots were separated on a 6% TBE-7M urea sequencing gel. The dried gel was exposed to Kodak BioMAX x-ray film at room temperature for approximately 18 hours.

Results from these five sets of reactions are shown in the photograph of the gel (Fig. 2), where each set are separately displayed, left to right, in the order 1 through 5 as described in the above table. Under conditions where neither ATP: sulfate adenylyltransferase nor inorganic pyrophosphatase were present in the

reactions (set 1), certain sequencing bands are either faintly or not visible as indicated by an asterisk on the figure. When ATP: sulfate adenylyltransferase is added (set 3), the bands are fully visible. In the presence of a lower concentration of this enzyme (set 4), only partial recovery of the bands is seen. Even though  
5 ATP: sulfate adenylyltransferase was present in set 5, the absence of the enzyme substrate adenosine 5'-phosphosulfate prevented the enzyme from reacting with pyrophosphate and providing protection from band loss. Since the preparation of ATP: sulfate adenylyltransferase used in this example was contaminated with  
10 deoxyribonucleases, the presence of some non-specific DNA fragment sizes (smearing) in the gel causes an increase in the background, but does not prevent the demonstration of the effectiveness of ATP: sulfate adenylyltransferase from removing pyrophosphate from the sequencing reactions. The inhibition of pyrophosphorolysis demonstrated in this example is dependent on both enzyme and enzyme substrate and is not the result of a contaminating pyrophosphatase-  
15 type activity. For reference, the action of inorganic pyrophosphatase in protecting from band loss is shown in set 2.

***Example 3 Use of Pyrophosphate: fructose-6-phosphate 1-phosphotransferase to eliminate pyrophosphorolysis in DNA sequencing***

20 Template and primer sufficient for 7 sequencing reactions were annealed in a 39  $\mu$ l reaction volume by incubating for two minutes at 65°C in a heating block and then slowly cooling the reaction to less than 37°C. The composition of the reaction was: 74 nM M13mpl9(+) strand DNA, 90 nM M13/pUC 23 base Forward Sequencing Primer, 360 mM Tris-HCl (pH 7.5), 90 mM NaCl, 36 mM MgCl<sub>2</sub>. Seven separate reactions were then radiolabeled by incubating the  
25 following 15.5  $\mu$ l reactions at room temperature (23-24°C) for 2 minutes: 26.5 nM M13mpl9(+) strand DNA, 32 nM M13/pUC 23 base Forward Sequencing Primer, 129 mM Tris-HCl (pH 7.5), 32 mM NaCl, 13 mM MgCl<sub>2</sub>, 6.5 mM DTT, 0.32  $\mu$ M (0.32  $\mu$ Ci/ $\mu$ l) [ $\alpha$ -<sup>35</sup>S]dATP, 0.194  $\mu$ M dCTP, 0.194  $\mu$ M 7-deaza-dGTP, 0.194  $\mu$ M dTTP, 0.21 U/ $\mu$ l Sequenase Version 2.0 T7 DNA Polymerase.

Additional components were present in the indicated reactions at the concentrations given in the following table:

Reaction Number	Concentration of Inorganic Pyrophosphate (U/ $\mu$ l)	Concentration of Fructose-6-phosphate* (mM)	Concentration of Fructose-2,6-diphosphate** ( $\mu$ M)	Concentration of Pyrophosphate: fructose-6-phosphate 1-phosphotransferase*** (U/ $\mu$ l)
1	0	0	0	0
2	0.00004	0	0	0
3	0	0.32	0.09	0.0032
4	0	0.32	0.09	0.00065
5	0	0.32	0.09	0.00032
6	0	0	0.09	0.0032
7	0	0	0.09	0.00065

\* Fructose-6-phosphate (Cat.No. F-3627) Sigma

\*\* Fructose-2,6-diphosphate (Cat.No. F-7006) Sigma

\*\*\* Pyrophosphate: fructose-6-phosphate 1-phosphotransferase, from Mung Bean (Cat.No.F-8757)

Each mixture was then divided into four tubes for completion of base-specific termination reactions. These sets of reactions were incubated for 30 minutes at 37°C and had the following compositions in 6  $\mu$ l reaction volumes: 15.4 nM M13mp19(+) strand DNA, 18.5 nM M13/pUC 23 base Forward Sequencing Primer, 75 mM Tris-HCl (pH 7.5), 40 mM NaCl, 7.5 mM MgCl<sub>2</sub>, 3.8 mM DTT, 0.19  $\mu$ M (0.19  $\mu$ Ci/ $\mu$ l) [ $\alpha$ -<sup>35</sup>S]dATP, 33.3  $\mu$ M dATP, 33.4  $\mu$ M dCTP, 33.4  $\mu$ M 7-deaza-dGTP, 33.4  $\mu$ M dTTP, 0.12 U/ $\mu$ l Sequenase Version 2.0 T7 DNA Polymerase, and 3.3  $\mu$ M ddATP, ddCTP, ddGTP or ddTTP. Additional components were present in the indicated reactions at the concentrations given in the following table:

Reaction Number	Concentration of Inorganic Pyrophosphatase (U/ $\mu$ l)	Concentration of Fructose-6-phosphate (mM)	Concentration of Fructose-2,6-diphosphate ( $\mu$ M)	Concentration of Pyrophosphate: fructose-6-phosphate 1-phosphotransferase (U/ $\mu$ l)
1	0	0	0	0
2	0.00002	0	0	0
3	0	0.19	0.05	0.0019
4	0	0.19	0.05	0.00038
5	0	0.19	0.05	0.00019
6	0	0	0.05	0.0019
7	0	0	0.05	0.00038

Reactions were stopped by adding 4  $\mu$ l of 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF and denatured for 2 minutes at 70°C. Three-microliter aliquots were separated on a 6% TBE-7 M urea sequencing gel. The dried gel was exposed to Kodak BioMAX x-ray film at room temperature for approximately 18 hours.

Results from these seven sets of reactions are shown in the photograph of the gel (Fig. 3), where each set are separately displayed, left to right, in the order 1 through 7 as described in the above table. Under conditions where neither Pyrophosphate: fructose-6-phosphate 1-phosphotransferase nor inorganic pyrophosphatase were present in the reactions (set 1), certain sequencing bands are either faintly or not visible as indicated by an asterisk on the figure. When Pyrophosphate: fructose-6-phosphate 1-phosphotransferase is added (set 3), the bands are fully visible. In the presence of lower concentrations of this enzyme (sets 4-5), only partial recovery of the bands is seen. Even though Pyrophosphate: fructose-6-phosphate 1-phosphotransferase was present at two different concentrations in sets 6 and 7, the absence of the enzyme substrate fructose-6-phosphate prevented the enzyme from reacting with pyrophosphate and providing protection from band loss. The inhibition of pyrophosphorolysis demonstrated in

the example is dependent on both enzyme and enzyme substrate and is not the result of a contaminating pyrophosphatase-type activity. For reference, the action of inorganic pyrophosphatase in protecting from band loss is shown in set 2.

5 From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions without undue experimentation. All patents, patent applications and publications cited herein are incorporated by reference in their entirety.

***What Is Claimed Is:***

1. A method of inhibiting or preventing pyrophosphorolysis during synthesis of a nucleic acid molecule, said method comprising

5 (a) combining one or more nucleotides and a nucleic acid template; and

(b) incubating the one or more nucleotides and nucleic acid template together with a polymerase and an enzyme selected from the group consisting of a pentosyltransferase, a phosphotransferase with alcohol group as acceptor, a nucleotidyltransferase, and a carboxy-lyase, under conditions sufficient to form a  
10 second nucleic acid molecule complementary to all or a portion of the nucleic acid template.

2. A method of inhibiting or preventing pyrophosphorolysis during synthesis of a nucleic acid molecule, said method comprising

15 (a) combining a primer with a nucleic acid template under conditions sufficient to form a hybridized product; and

(b) incubating said hybridized product in the presence of (i) one or more nucleotides, (ii) a polymerase, and (iii) an enzyme selected from the group consisting of a pentosyltransferase, a phosphotransferase with an alcohol group as acceptor, a nucleotidyltransferase, and a carboxy-lyase under conditions  
20 sufficient to synthesize a second nucleic acid molecule complementary to all or a portion of said nucleic acid template.

3. The method of claim 2, wherein said enzyme of (b)(iii) is a pentosyltransferase.

4. The method of claim 3, wherein said enzyme is an adenine  
25 phosphoribosyltransferase or an orotate phosphoribosyltransferase.



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5. The method of claim 2, wherein said enzyme of (b)(iii) is a phosphotransferase with an alcohol group as acceptor.

6. The method of claim 5, wherein said enzyme is a pyrophosphate: glycerol phosphotransferase, a pyrophosphate: serine phosphotransferase, a  
5 pyrophosphate: fructose-6-phosphate 1-phosphotransferase or a pyrophosphate: purine nucleoside kinase.

7. The method of claim 2, wherein said enzyme of (b)(iii) is a nucleotidyltransferase.

8. The method of claim 7, wherein said enzyme is an ATP: sulfate  
10 adenyltransferase, a UTP: glucose-1-phosphate uridylyltransferase or an ATP: glucose-1-phosphate adenyltransferase.

9. The method of claim 2, wherein said enzyme of (b)(iii) is a carboxy-lyase.

10. The method of claim 9, wherein said enzyme is a  
15 phosphoenolpyruvate carboxykinase.

11. The method of claim 2, wherein said enzyme of (b)(iii) is a thermostable enzyme.

12. The method of claim 2, wherein said nucleotide is a deoxyribonucleoside triphosphate and said polymerase is a DNA polymerase.

13. The method of claim 1, wherein said nucleotide is a ribonucleoside  
20 triphosphate and said polymerase is an RNA polymerase.

14. A method to prevent inhibition of nucleic acid synthesis during amplification of a double stranded nucleic acid molecule, comprising

5 (a) providing a first and second primer, wherein said first primer is complementary to a sequence at or near the 3' termini of the first strand of said nucleic acid molecule and said second primer is complementary to a sequence at or near the 3' termini of the second strand of said nucleic acid molecule;

10 (b) hybridizing said first primer to said first strand and said second primer to said second strand in the presence of (i) a polymerase, and (ii) an enzyme selected from the group consisting of a pentosyltransferase, a phosphotransferase with an alcohol group as an acceptor, a nucleotidyltransferase and a carboxy-lyase under conditions such that a third nucleic acid molecule complementary to said first strand and a fourth nucleic acid molecule complementary to said second strand are synthesized;

15 (c) denaturing said first and third strand and said second and fourth strand; and

(d) repeating steps (a) to (c) one or more times.

15. The method of claim 14, wherein said enzyme of (b)(ii) is a pentosyltransferase.

20 16. The method of claim 15, wherein said enzyme is an adenine phosphoribosyltransferase or an orotate phosphoribosyltransferase.

17. The method of claim 14, wherein said enzyme of (b)(ii) is a phosphotransferase with an alcohol group as acceptor.

25 18. The method of claim 17, wherein said enzyme is a pyrophosphate: glycerol phosphotransferase, a pyrophosphate: serine phosphotransferase, a pyrophosphate: fructose-6-phosphate 1-phosphotransferase or a pyrophosphate: purine nucleoside kinase.

19. The method of claim 14, wherein said enzyme of (b)(ii) is a nucleotidyltransferase.

20. The method of claim 19, wherein said enzyme is an ATP: sulfate adenylyltransferase, a UTP: glucose-1-phosphate uridylyltransferase or an ATP: glucose-1-phosphate adenylyltransferase.

21. The method of claim 14, wherein said enzyme of (b)(ii) is a carboxy-lyase.

22. The method of claim 21, wherein said enzyme is a phosphoenolpyruvate carboxykinase.

23. The method of claim 14, wherein said enzyme of (b)(ii) is a thermostable enzyme.

24. The method of claim 14, wherein said polymerase is a DNA polymerase.

25. A method of sequencing a DNA molecule comprising:

(a) combining a primer with a first DNA molecule under conditions sufficient to form a hybridized product;

(b) contacting said hybridized product with (i) nucleotides; (ii) a DNA polymerase; (iii) an enzyme selected from the group consisting of a pentosyltransferase, a phosphotransferase with an alcohol group as acceptor, a nucleotidyltransferase and a carboxy-lyase; and a terminator nucleotide to give a reaction mixture;

(c) incubating the reaction mixture under conditions sufficient to synthesize a random population of DNA molecules complementary to said first DNA molecule, wherein said synthesized DNA molecules are shorter in length

than said first DNA molecule and wherein said synthesized DNA molecules comprise a terminator nucleotide at their 3' termini; and

(d) separating said synthesized DNA molecules by size so that at least a part of the nucleotide sequence of said first DNA molecule can be determined.

5           26. The method of claim 25, wherein said enzyme of (b)(iii) is a pentosyltransferase.

27. The method of claim 26, wherein said enzyme is an adenine phosphoribosyltransferase or an orotate phosphoribosyltransferase.

10           28. The method of claim 25, wherein said enzyme of (b)(iii) is a phosphotransferase with an alcohol group as acceptor.

29. The method of claim 28, wherein said enzyme is a pyrophosphate: glycerol phosphotransferase, a pyrophosphate: serine phosphotransferase, a pyrophosphate: fructose-6-phosphate 1-phosphotransferase or a pyrophosphate: purine nucleoside kinase.

15           30. The method of claim 25, wherein said enzyme of (b)(iii) is a nucleotidyltransferase.

31. The method of claim 30, wherein said enzyme is an ATP: sulfate adenylyltransferase, a UTP: glucose-1-phosphate uridylyltransferase or an ATP: glucose-1-phosphate adenylyltransferase.

20           32. The method of claim 25, wherein said enzyme of (b)(iii) is a carboxy-lyase.

33. The method of claim 32, wherein said enzyme is a phosphoenolpyruvate carboxykinase.

34. The method of claim 25, wherein said enzyme of (b)(iii) is a thermostable enzyme.

5           35. The method of claim 25, wherein said nucleotides are deoxyribonucleoside triphosphates and said polymerase is a DNA polymerase.

36. A solution for use in nucleic acid synthesis, amplification or sequencing, comprising

10           (a) an enzyme selected from the group consisting of a pentosyltransferase, a phosphotransferase with alcohol group as acceptor, a nucleotidyltransferase, and a carboxy-lyase;

            (b) a substrate which is capable of either accepting either a phosphate radical to give a phosphorylated product from pyrophosphate or effecting transfer of pyrophosphate when in the presence of said enzyme; and

15           (c) a polymerase.

37. The solution of claim 36, wherein said enzyme of (a) is a pentosyltransferase.

38. The solution of claim 37, wherein said enzyme is an adenine phosphoribosyltransferase or an orotate phosphoribosyltransferase.

20           39. The solution of claim 36, wherein said enzyme of (a) is a phosphotransferase with an alcohol group as acceptor.

40. The solution of claim 39, wherein said enzyme is a pyrophosphate: glycerol phosphotransferase, a pyrophosphate: serine phosphotransferase, a

pyrophosphate: fructose-6-phosphate 1-phosphotransferase or a pyrophosphate: purine nucleoside kinase.

41. The solution of claim 36, wherein said enzyme of (a) is a nucleotidyltransferase.

5           42. The solution of claim 41, wherein said enzyme is an ATP: sulfate adenylyltransferase, a UTP: glucose-1-phosphate uridylyltransferase or an ATP: glucose-1-phosphate adenylyltransferase.

43. The solution of claim 36, wherein said enzyme of (a) is a carboxy-lyase.

10           44. The solution of claim 43, wherein said enzyme is a phosphoenolpyruvate carboxykinase.

45. The solution of claim 36, wherein said enzyme of (a) is a thermostable enzyme.

15           46. The solution of claim 36, wherein said polymerase is a DNA polymerase.

47. The solution of claim 36, wherein said polymerase is an RNA polymerase.

20           48. A kit comprising a container means having in close confinement therein two or more container means, wherein a first container means comprises an enzyme selected from the group consisting of a pentosyltransferase, a phosphotransferase with an alcohol group as acceptor, a nucleotidyltransferase, and a carboxy-lyase; and a third container means contains a substrate which is

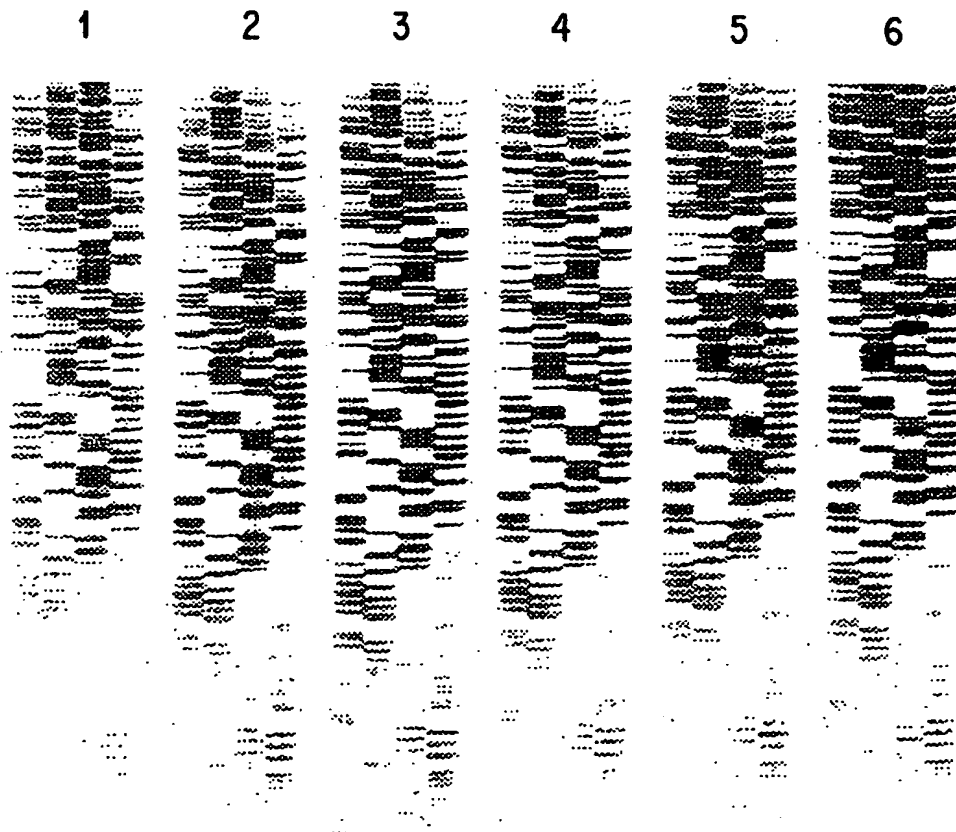
capable of either accepting a phosphate radical to give a phosphorylated product from pyrophosphate or effecting transfer of pyrophosphate when in the presence of said enzyme;

5 wherein a nucleic acid polymerase is optionally present in said first container means or is optionally comprised in a second container means.

49. The kit of claim 48, wherein said nucleic acid polymerase is present in said first container means.

50. The kit of claim 48, wherein said nucleic acid polymerase is present in said second container means.

10 51. The kit of claim 48, wherein said enzyme is a thermostable enzyme.



ACGT

FIG. 1  
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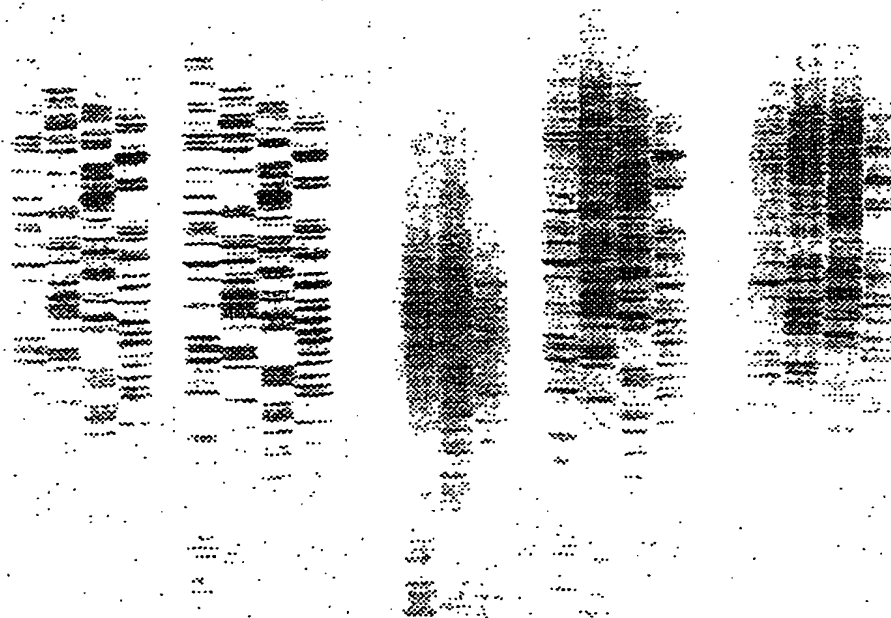


FIG.2  
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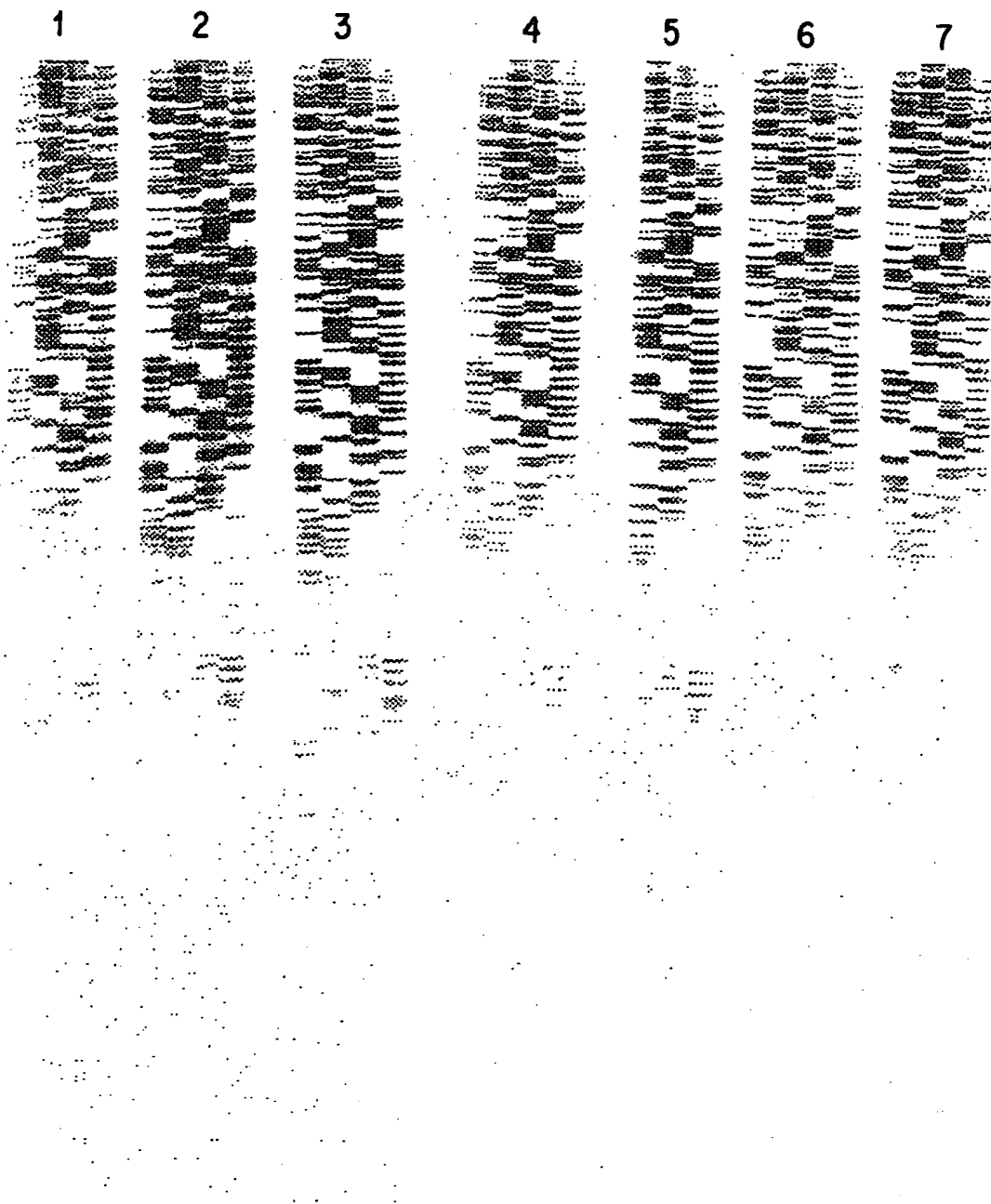


FIG.3

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/21037

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12P 19/34  
US CL : 435/91.1, 91.2

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/91.1, 91.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, CA, DERWENT

search terms: pyrophosphorolysis, pyrophosphate, sequencing, amplification, PCR

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Nyren. Enzymatic method for continuous monitoring of DNA polymerase activity. Analytical Biochemistry. 1987, Vol. 167, pages 235-238, especially page 236.	1, 2, 7, 8, 12, 36, 41, 42, 46 ----- 3-6, 9-11, 13-35, 37- 40, 43-45, 47-51
Y	US 5,498,523 A (TABOR et al.) 12 March 1996, col. 2, lines 18-47 and column 3, lines 40-67.	1-51
Y	US 4,962,020 A (TABOR et al.) 09 October 1990, col. 16, lines 6-33.	1-51

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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